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CHAPTER 36
Dendritic Cell-derived Exosomes: Potent Immunogenic Cell-free Vaccines

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INTRODUCTION

Antigen-presenting cells (APC) contain a specialized late endocytic compartment, MIIC (MHC class II-enriched compartment), that harbors newly synthesized MHC class II molecules in transit to the plasma membrane (Nijman, 1995; Kleijmeer, 1995). MIIC have lysosomal characteristics (they are acidic and bear lysosomal marker molecules, i.e., LAMP, tetraspanins) and are involved in antigen processing and peptide binding to class II molecules. However, functionally different subclasses of MIIC exist, encompassing membrane sheet and/or internal vesicle (multivesicular bodies, MVB) containing compartments. Ultrastructural studies of EBV-transformed B cells demonstrate that multivesicular bodies are exocytic compartments in that their limiting external membrane can fuse with the plasma membrane resulting in the release, into the extracellular milieu, of their internal vesicular content. The externalized vesicles, termed exosomes, carry in their membrane MHC class II molecules with their peptide-binding domain oriented toward the extracellular milieu. During their formation, internal vesicles arise from the budding of a portion of the outer endosomal membrane toward the endosomal lumen (Raposo *et al.*, 1997; Fig. 1). These MHC class II molecules are functional and induce antigen-specific MHC class II-restricted T cell responses *in vitro* (Raposo *et al.*, 1996). Interestingly, Kleijmeer *et al.* (1998) reported that MHC class I molecules colocalize with MHC class II and tetraspanin molecules in the external membrane and internal vesicles of these MVB.

We now show that dendritic cells (DC) secrete antigen-presenting vesicles in a regulated manner. Importantly, tumor peptide pulsed DC-derived exosomes mediate potent MHC-dependent anti-tumor immune responses that induce tumor rejection in mice (Zitvogel *et al.*, 1998).

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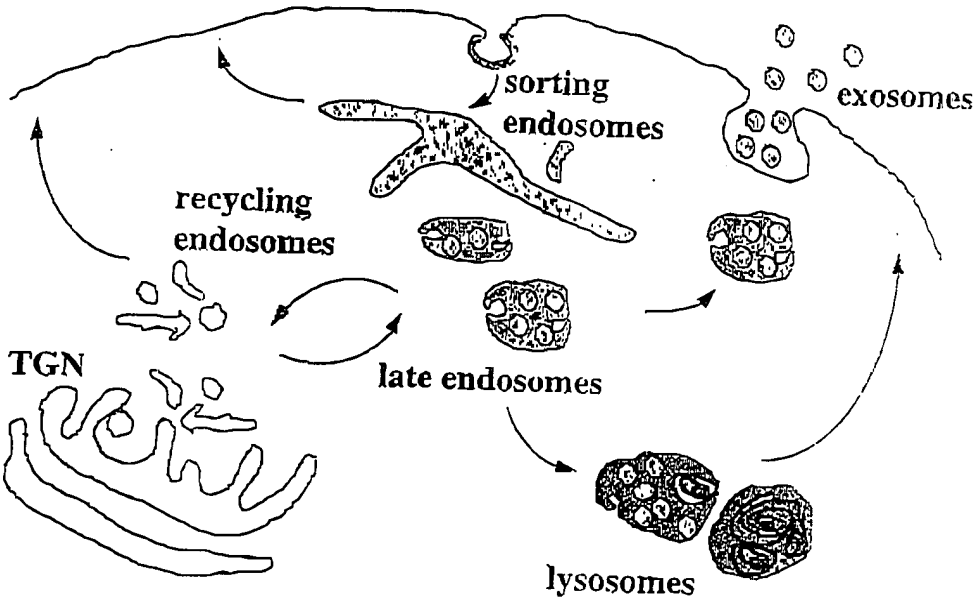

Fig. 1. Schematic representation of the endocytic and exocytic system of antigen-presenting cells. Ultrastructural studies have revealed that the vast majority of MHC class II molecules reside in lysosome-related late endocytic compartments, the MIIC. MIIC encompass both multilamellar-containing compartments and/or multivesicular bodies (MVB). MVB represent a meeting point between the endocytic and the exocytic pathways. At the electron-microscopic level, the late endosome appears as a 200–300 nm membrane compartment containing in its lumen variable amounts of small 60–90 nm vesicles. The internal vesicles of the MVB are thought to arise from budding of a portion of the limiting membrane into the endosomal lumen. During the invagination toward the intraluminal milieu, some membrane proteins are sequestered in the internal vesicles, whereas others remain in the limiting membrane of the MVB. The external membrane of MVB fuses with the plasma membrane, resulting in the exocytosis of the internal vesicular content, the exosomes, into the extracellular milieu. TGN, trans-Golgi network.

HUMAN AND MOUSE DC SECRETE THE INTERNAL VESICLES OF MULTIVESICULAR LATE ENDOSOMES

We first examined secretory lysosomes from immature human monocyte-derived DC cultured in IL-4 + GM-CSF. Ultrastructural studies revealed that these cells contain numerous internal vesicles (multivesicular MIIC) as well as MIIC displaying electron-dense concentrically arranged membrane sheets (multilamellar MIIC). As described by Kleijmeer *et al.* (1998) in EBV-transformed B cells, we also found that in human monocyte-derived (MD)-DC generated in IL-4 + GM-CSF for 7–10 days, multivesicular MIIC express MHC class I molecules (Zitvogel *et al.*, 1998). Both markers were found in the external membrane of the endosomes and the intraluminal 60–90 nm vesicles (not shown). Multilaminar compartments were not labeled with anti-MHC class I antibodies (not shown). In contrast to CD63 (Metzelaar, 1991) or CD82, MHC class I molecules were also detected at the cell surface (Zitvogel *et al.*, 1998). Multivesicular MHC class I- and class II-containing compartments were often observed

in close apposition to the cell surface, suggesting their direct fusion with the plasma membrane (Zitvogel *et al.*, 1998). Consistent with this possibility, 60–90 nm vesicles were often observed close to the outer side of the plasma membrane. These vesicles were abundantly labeled with anti-MHC class I and II, CD63, and CD82 specific antibodies. Therefore, 60–90 nm vesicles, bearing the same markers as the internal vesicles of multivesicular MIIC (class I, class II, CD63, CD82) are released by human DC.

These vesicles were isolated from DC culture supernatants following differential ultracentrifugation (Raposo *et al.*, 1996) and analyzed by whole-mount immunoelectron microscopy. A homogeneous population of vesicles of 60–90 nm diameter was observed (Fig. 2, lower panel). Like the vesicles from the exocytic profiles, over 95% of these vesicles were labeled with the anti-CD63 and anti-CD82 antibodies, as well as with anti-MHC class I and class II antibodies (not shown).

Similar vesicles were observed in the supernatants of mouse DC. We used the well-characterized growth factor-dependent D1 DC line (Winzler *et al.*, 1997) and bone marrow-derived DC (BM-DC) to analyze the exosomal production by mouse DC. As in the case of human DC, immature murine DC exhibit abundant multivesicular endosomal compartments (Fig. 2, upper panel), which were occasionally observed in close apposition to the plasma membrane. This Lamp-1 positive endosomal population stained for MHC class I and II molecules by confocal immunofluorescence (data not shown). Exosomes, harvested from D1 (Fig. 2, lower panel) or BM-DC supernatants, expressed MHC class I, class II (Fig. 2, upper panel) and costimulatory signal molecules as detected by whole-mount electron microscopy and Western blot (not shown). MHC class I and class II as well as CD86 and transferrin receptors (TfRs) were found on exosomes; the three latter markers were enriched in exosomes as compared with the cell lysates. In contrast, although detected in the cell lysates, H2-M, Ii chain, and calnexin (an endoplasmic reticulum-specific marker) were undetectable in the exosomal preparations. The size and morphology of immature mouse DC-derived exosomes were similar to the size and morphology of those derived from human DC.

EXOSOMES HAVE ALLOSTIMULATORY CAPACITIES AND ARE MHC CLASS I-RESTRICTED ANTIGEN PRESENTING VESICLES *IN VITRO*

Allogeneic lymphocytes were capable of proliferating when cocultured with DC-derived exosomes in a day 5 *in vitro* thymidine incorporation assay. Their proliferation rate was 50 times greater when whole irradiated DC were used as stimulators (not shown).

To determine whether exosomes may directly stimulate a $CD8^+$ cytotoxic, HLA-A2-restricted T cell clone (Duffour *et al.*, 1997), HLA-A2-positive human monocyte-derived DC were pulsed with MART-1/MelanA$_{(27-35)}$ peptides and exosomes were isolated from the cell culture supernatants.

The MART-1/MelanA-pulsed DC-derived exosomes were capable of stimulating IFN-γ production of a MART-1/MelanA-specific HLA-A2-restricted CTL clone LT12 in a dose-dependent manner. Exosomes produced by DC pulsed with a control peptide (gp100$_{(280-288)}$) had no stimulatory effect on this clone. Thus, MHC class I molecules displayed at the surface of DC-derived exosomes are functional but to a lesser extent than the cells from which they are secreted (Zitvogel *et al.*, 1998).

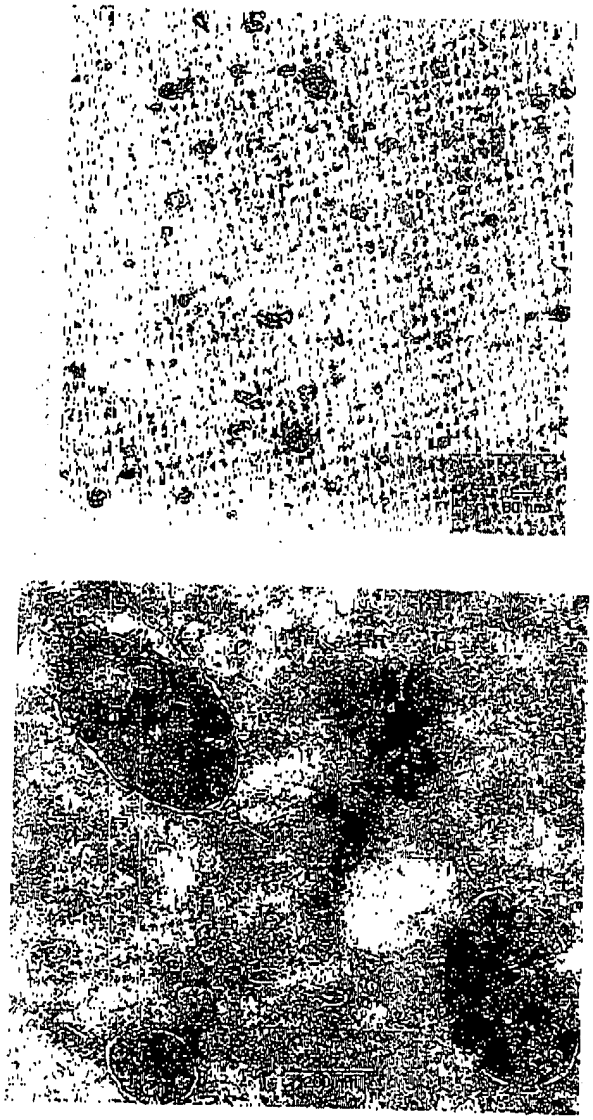

Fig. 2. MHC class II compartments of mouse D1 DC. Upper panel: Ultrathin cryosections of D1 cells were immunogold labeled for MHC class II (protein A–10 nm gold). MHC class II are localized in compartments displaying internal membrane vesicles (MVB). Lower panel: Whole-mount electron microscopy of D1-derived exosomes. The 100 000*g* pellets obtained after differential ultracentrifugation of D1 supernatants are composed of small vesicles with diameter varying from 50 to 90 nm. Bars: 250 nm.

TUMOR PEPTIDE-PULSED DC-DERIVED EXOSOMES INDUCE TUMOR GROWTH SUPPRESSION IN TUMOR-BEARING MICE

We tested the capacity of these vesicles to induce T cell-mediated immune responses *in vivo*. Bone marrow-derived DC cultured in IL-4 + GM-CSF (BM-DC) (Mayordomo *et al.*, 1995; Zitvogel *et al.*, 1996), loaded with acid-eluted tumor peptides, were

previously shown to mediate specific anti-tumor immune responses. P815 is an immunogenic but aggressive mastocytoma, syngeneic for DBA/2 (H-2^d), for which very few effective immunotherapies on day-10 established tumors have been reported. Acid-eluted tumor (P815) peptides were pulsed onto syngeneic mouse BM-DC as previously described (Zitvogel *et al.*, 1996). Exosomes were prepared from the DC supernatants by differential ultracentrifugation and utilized for *in vivo* immunization.

As shown in Fig. 3A, therapy of day-10 established P815 tumors (50–90 mm^2 in size) was carried out using a single intradermal (i.d.) administration of 3–5 μg of exosomes per mouse. Within a week, tumor growth stopped in the groups receiving exosomes derived from autologous tumor peptide-pulsed DC and 40–60% mice were tumor free at day 60 (Fig. 3A). These animals had a long-lasting immune response and rejected a lethal tumor challenge with P815 but not with the syngeneic leukemia L1210 (not shown). Groups of mice immunized with exosomes derived from self splenic peptide-pulsed DC showed no effect on tumor growth as compared with control mice groups (Fig. 3A). Thus, P815 peptide-pulsed DC-derived exosomes promoted tumor regression.

Similar anti-tumor effects were achieved in the day 3–4 established TS/A tumor model. These anti-tumor effects were not found in athymic Nu/Nu counterparts, indicating that T cells are required for the exosome-induced anti-tumor immune responses. In addition, exosomes directly prime tumor-specific CTL responses in P815-bearing hosts. Splenocytes from mice that rejected P815 tumors following immunization with exosomes were harvested at day 90 and cultured for 5 days in the presence of irradiated B7.1-expressing P815 cells to enhance specific precursor frequency. These effector cells were tested in a 4 h ^{51}Cr-release assay against the autologous tumor cells P815 (H-2^d), against the irrelevant leukemia (H-2^d) L1210, and against YAC cells. Significant specific lytic activity on P815 was achieved in splenocytes from exosome-immunized mice (not shown). Interestingly, none of the spleens from littermates spontaneously rejecting P815 or bearing growing P815 tumors displayed cytolytic activity against P815 under the same conditions (not shown). Therefore, a single injection of exosomes derived from DC pulsed with the relevant peptides efficiently primed specific antitumor CTL responses *in vivo*.

Exosomes Induce MHC-Restricted Response

To determine whether exosome-induced immune responses are MHC restricted and not simply due to any direct effect of acid-eluted tumor peptides, day-5 BM-DC derived from H-2^d (DBA/2) or H-2^b (C57BL/6) mice were pulsed in parallel with acid-eluted P815 tumor peptides. Exosomes were then isolated and separately utilized for direct i.d. injection of DBA/2 mice bearing 6–10-day established P815 tumors. Only the syngeneic tumor peptide-bearing exosomes were potent tumor vaccines (with up to 60% tumor-free mice), whereas the allogeneic counterparts did not promote significant anti-tumor effects. These results suggest that these antigen-presenting vesicles induce MHC-restricted anti-tumor effects *in vivo* (Fig. 3A). When the exosomes derived from BM-DCs (DBA/2) pulsed with the tumor-specific subdominant epitope P1A were injected i.d. to protect mice against subsequent challenge with P815, up to 75% of mice were efficiently capable of preventing the onset of the tumor, whereas control peptide

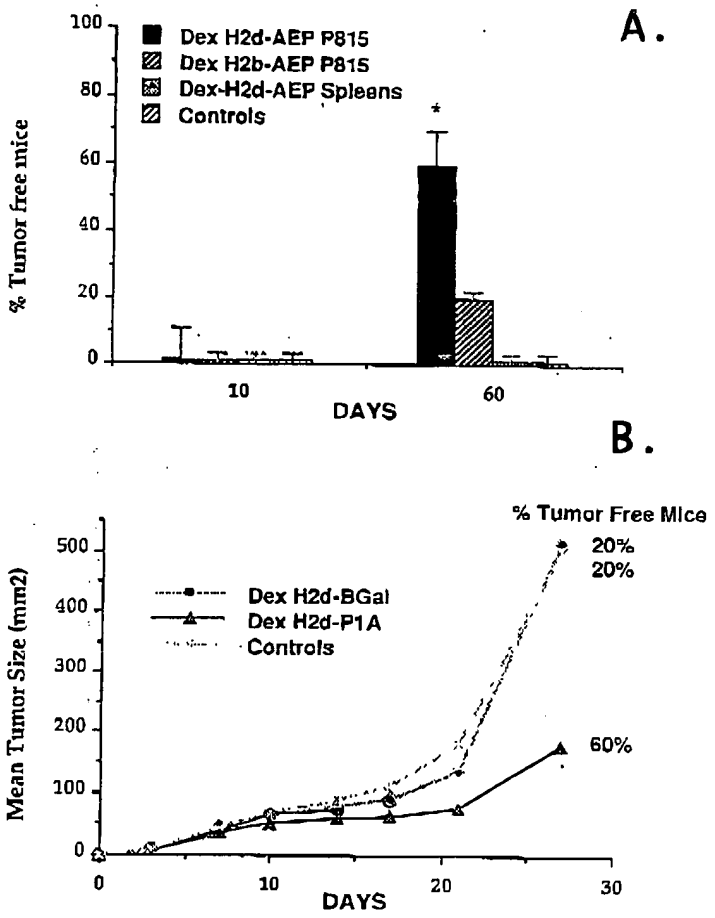

Fig. 3. Anti-tumor effects following administration of exosomes derived from tumor peptide-pulsed BM-DC. (A) DBA/2 animals bearing 50–80 mm^2 tumors were immunized intradermally in the lower part of ipsilateral flank with exosomes (3–5 μg/mouse) from BM-DC $H2^d$ pulsed with acid-eluted P815 tumor peptides ($DexH2^d$-AEP P815) or with acid-eluted DBA/2 spleen peptides ($DexH2^d$-AEP Spleens) or with acid-eluted P815 tumor peptides ($DexH2^b$-AEP P815). Tumor sizes were monitored twice a week. The percentages of tumor-free mice at days 10 and 60 are represented. Five DBA/2 mice per group were vaccinated and two subsequent experiments were performed with similar results. (B) Exosomes derived from BM-DC DBA/2 pulsed with the subdominant P1A tumor epitope were injected i.d. 15 days prior to tumor challenge with a lethal dose of P815 tumor cells. Control groups were protected either with saline or with exosomes derived from BM-DC DBA/2 pulsed with the H2d-BGal epitope TPHPARIGL. The percentage of tumor-free mice at day 60 is represented as well as the tumor growth over time.

(BGal, H2d)-bearing exosomes were not efficient (Fig. 3B). Importantly, intravenous or i.d. adoptive therapy using $5–10 \times 10^5$ immature BM-DC pulsed with acid-eluted tumor peptides was not as efficient as i.d. administration of exosomes derived from the same cells (Fig. 4) in curing mice. Similar results were achieved on established TS/A tumors in BALB/c animals and on prophylaxis studies using the P1A tumor peptide (not shown).

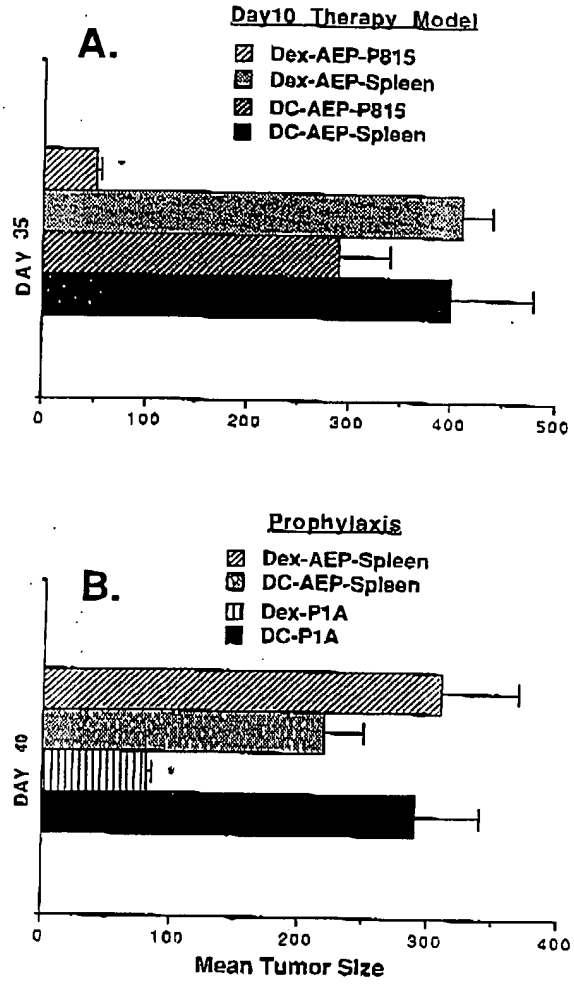

Fig. 4. Exosomes are more potent than immature dendritic cells, from which they derive, in eradicating established tumors *in vivo*. (A) DC (5×10^6) pulsed with either acid-eluted splenic peptides (AEP) or P815 tumor peptides were administered i.v. or i.d. at days 8–10 following P815 i.d. establishment in 5 mice. In parallel, the supernatants of these cells were harvested following 18 h incubation with splenic peptides or P815 relevant peptides, ultracentrifuged, and characterized for exosome production (DC exosomes, Dex). Up to 5 μg/5×10^6 DC allow i.d. immunization of 5 mice in the ipsilateral flank. A single administration of exosome was performed at day 8–10. The mean tumor size at day 35 is depicted. * represents significant results at 95% using Fisher's exact method compared with injection of saline or peptide-pulsed DC. Representative data are shown among additional similar experiments. (B) Similar experiments but DC or Dex were injected as prophylaxis, 15 days prior to tumor challenge with P815. P1A is used instead of acid-eluted tumor peptide in this setting. The mean tumor size at day 40 is depicted. * represents significant results at 95% using Fisher's exact method compared with injection of peptide-pulsed DC.

DISCUSSION

Ultrastructural studies of the possible routes of transport of lysosomal constituents to the cell surface in not only B cells but also DC revealed that late endosomal compartments, i.e., multivesicular bodies displaying intraluminal membrane vesicles, can fuse with the plasma membrane in an exocytic fashion and release, in the extracellular environment, 60–90 nm exosomes (Raposo, 1996; Raposo, 1997; Kleijmeer, 1998). We report that antigen-loaded DC-derived exosomes bear functional class I molecules that allow CTL priming *in vivo* and established tumor growth suppression in various murine experimental model systems (Zitvogel *et al.*, 1998).

Even though exosome release has been associated with clearance of transferrin receptors, reticulocyte maturation and differentiation into an erythrocyte pathway (Bockxmeer, 1979), the physiological relevance of exosome secretion and function *in vivo* are still a matter of debate. Our data imply an immunostimulatory role of this exosome release and show that immature mouse and human dendritic cells secrete exosomes in a regulated manner (unpublished data) that bear not only MHC class II molecules but also MHC class I as well as costimulatory signal molecules and are immunogenic in tumor-bearing mice.

Exosomes can be obtained in relatively high quantities (2–5 $\mu g/10^6$ DC per 18 h using the Bradford assay) from the culture media of immature DC (d5 mouse BM-DC in GM-CSF + IL-4, growth factor-dependent D1 line in the absence of activating stimuli, or CD83-negative MD-DC from human PBMC) following ultracentrifugation at 100 000*g* of the culture supernatants. We characterized exosomes by morphological and biochemical criteria. The membranes pelleted at 100 000*g*, analyzed by immuno-electron microscopy, represented a homogeneous population of vesicles that resembled those described for EBV-transformed B cells, labeling for MHC class I, class II, CD86, and lysosomal-associated tetraspan molecules (Metzelaar, 1991). Exosomes abundantly overexpressed MHC class II, tetraspanins such as CD63/CD82, and CD86 molecules as compared with plasma membrane. Endoplasmic reticulum markers were not detected in western blotting using anti-calnexin antibodies (Zitvogel *et al.*, 1998). Exosome preparations were apparently devoid of retroviruses, plasma membrane shedding, microsome constituents or apoptotic bodies. Interestingly, we reproducibly reduced the amounts of vesicles secreted by inducing maturation of the mouse DC D1 line or BM-DC, as assessed by Bradford assay, western blotting with MHC class I antibodies, and immuno-electron microscopy (unpublished data). The basal secretion can be further and significantly enhanced by lowering the culture pH or incubating with defined cytokines, strongly suggesting that exocytosis of antigen-presenting vesicles by DC is regulated.

The striking observation is that immature DC, purportedly considered to be poor antigen-presenting cells (Cella, 1997), are actually capable of secreting antigen-presenting exosomes that account for efficient T cell priming *in vivo*. Indeed, intradermal injection of a single dose of exosome derived from 5×10^5 tumor peptide-pulsed DC was associated with tumor growth suppression in established mammary or mastocytoma tumor models. DC-derived exosomes are more effective in prophylaxis and therapy of mouse tumors than whole DC-based vaccines. Indeed, 2–5 μg of exosomes secreted from 10^6 DC pulsed with tumor peptides were more protective or curative than the DC themselves. Although not yet biochemically defined, exosomes bear high

amounts of MHC class II, tetraspanins, and costimulatory molecules compared with DC lysates. The lipid composition of these compartments may be different from that of the plasma membrane and facilitates their *in vivo* uptake and/or transport to T cell-enriched areas. *Ex vivo* expanded DC represent valuable immunotherapeutic options for cancer-bearing patients (Steinman, 1991, 1996; Girolomoni, 1997) but phenotypic changes (surface markers, migration pathways, etc.) can be anticipated following withdrawal from the culture medium (Bender, 1996). In addition, MHC class I molecules on mature DC seem to have a relatively short half-life compared with MHC class II molecules (Cella, 1997). However, further molecular characterization of their composition and further investigation of their mechanisms are needed to better understand their *in vivo* efficacy.

The physiological role of DC-derived exosomes remains unclear. It is conceivable that T-helper cytokines are delivered to the DC upon arrival in the lymph node T cell-enriched areas. Antigen-presenting vesicles would then be released to amplify specific T cell clonal expansion. Alternatively, other host APC could take up these exosomes to transport these antigenic vesicles to specific sites where priming of naive T cells and/or B cell cross-talk could be elicited.

These data support the implementation of DC-derived exosomes for cancer immunotherapy as a novel dendritic cell-free therapeutic cancer vaccine and suggest that exosomes may represent a physiological means of communication between DC and T-lymphocytes.

SUMMARY

Dendritic cells (DC) are professional antigen-presenting cells having a unique ability to induce primary immune responses *in vitro* and *in vivo* (Inaba *et al.*, 1990; Caux *et al.*, 1992; Hart, 1997). Here, we show that antigen-presenting vesicles from endosomal MHC class II-enriched compartments can be secreted by DC and induce potent T cell-mediated anti-tumor immune responses *in vivo*. These vesicles, called 'exosomes', represent the internal vesicles of multivesicular endosomes, which are secreted following fusion of the external membrane of endosomes with the plasma membrane (Raposo *et al.*, 1996). Exosomes harvested from immature DC culture supernatants by ultracentrifugation were characterized by immuno-electron microscopy and western blotting. These vesicles harbor not only endosomal markers absent from the cell surface, but also express high levels of major histocompatibility complex (MHC) class I, class II, and costimulatory molecules. MHC class I molecule-bearing exosomes stimulate antigen-specific $CD8^+$ T cell clones *in vitro*. Importantly, intradermal injection of tumor peptide-pulsed DC-derived exosomes is capable of priming specific cytotoxic T-lymphocytes *in vivo* and suppressing growth of day 3–10 established murine tumors in a T cell-dependent and MHC-restricted manner. Cell-free-based exosome vaccines may be superior to dendritic cell adoptive therapy for controlling tumor growth. The anti-tumor effects of DC-derived exosomes support their implementation for cancer immunotherapy and suggest that exosomes represent a novel 'liposome-like' means of communication between cells of the immune system.

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